New Biotechnology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

New Biotechnology

journal homepage: www.elsevier.com/locate/nbt

Self-assembly and drug release study of linear L,D-oligopeptide-poly(ethylene glycol) conjugates

Federica Novelli[a], Serena De Santis[a], Pasqualina Punzi[a,1], Cesare Giordano[b], Anita Scipioni[a], Giancarlo Masci[a,*]

[a] Department of Chemistry, Sapienza University, P.le A. Moro, 5–00185 Rome, Italy
[b] Institute of Molecular Biology and Pathology, CNR, Sapienza University, P.le A. Moro, 5–00185 Rome, Italy

ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form 22 June 2016
Accepted 15 July 2016
Available online xxx

Keywords:
Linear L,D-peptide
Poly(ethylene glycol)
Conjugated L,D-peptide-polymer
Self-assembly
Nanoparticles
Drug loading
Sustained release

ABSTRACT

The preparation and structural organisation of new bioinspired nanomaterials based on regular alternating enantiomeric sequence of tetra- and hexapeptides end-linked to poly(ethylene glycol) (PEG) is reported. The peptide moiety is composed of two or three repeats of L-Ala-D-Val units while the PEG has a molecular weight of 2 kDa. The self-assembling properties of the two conjugates depend significantly on the length of the peptide. Nanoparticles with different sizes and morphologies are formed, the structural properties of which are compared with the previously studied L-Ala-D-Val octapeptide conjugate that self-assembles into rod-like nanoparticles. The aggregation properties were studied by NMR, circular dichroism, fluorescence spectroscopies and dynamic light scattering. The morphology and size of the nanoparticles were assessed by scanning electron microscopy and dynamic light scattering. The loading and release of a model drug were also investigated. This study demonstrates that, by changing the length of the peptide, it is possible to modulate the self-assembly and loading properties of peptide-PEG conjugates.

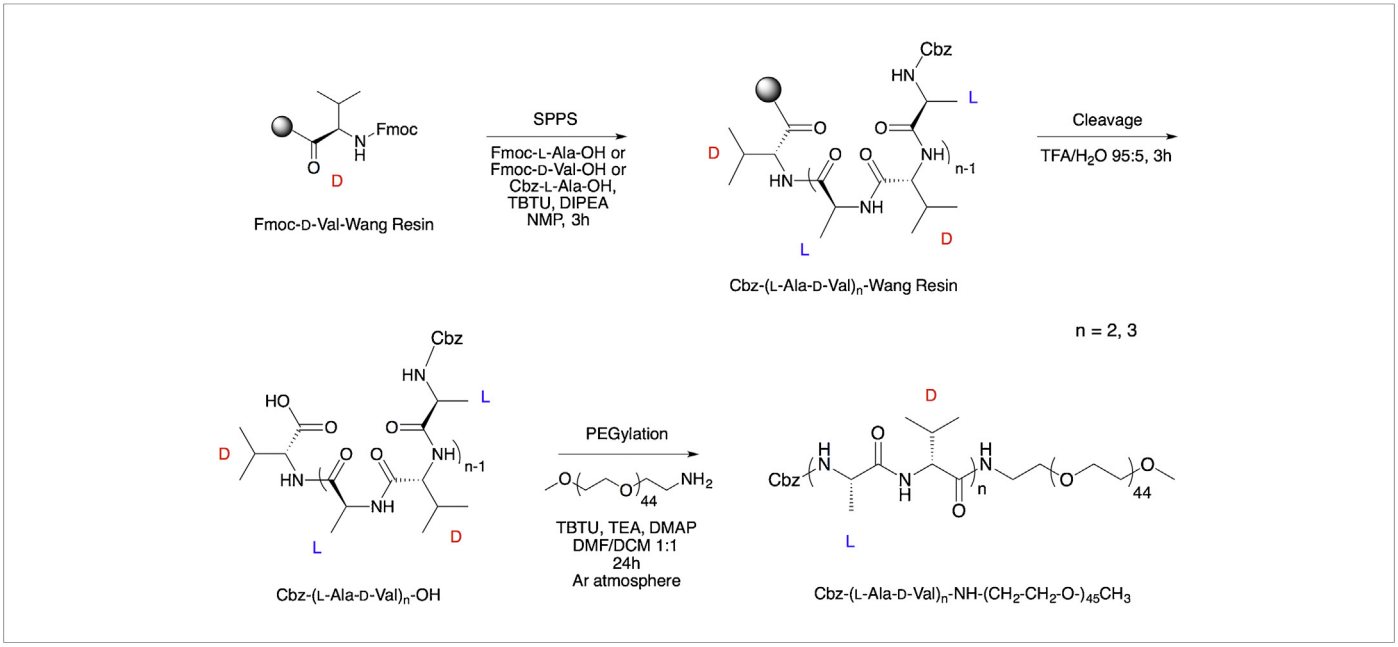
Introduction

In recent years, self-assembly of small molecules or polymers has emerged as a powerful bottom-up approach for the preparation of new materials with defined architectures and functionalities [1]. Among these conjugates, oligopeptides have been used extensively as building blocks to achieve biologically inspired nanoparticles with various applications ranging from medicine to electronics, because amino acid side chain functionalities allow tuning f the properties of the final nano-structure [2–5]. Peptides formed by the linear L,D enantiomeric sequences formed of conformationally equivalent residues are particularly interesting to obtain self-assembling nanoparticles. Theoretical [6] and experimental aspects [7–11] of such peptides were investigated in depth by De Santis et al. who first demonstrated that they can provide low-pitch helices with an almost planar structure that can self-assemble into tubular aggregates stabilised by hydrogen bonds and van der Waals interactions [6]. Ghadiri et al. [12] first proposed the use of cyclic peptides with regularly alternating configurations to obtain self-assembling nanoparticles. The surface properties of these structures can be modulated by changing the peptide sequences or modifying the functional groups of the amino acid side chains by linking to suitable synthetic macromolecules in order to obtain nanoparticles with applications in different fields [13–18].

When a hydrophobic peptide is conjugated to a hydrophilic polymer, the morphology and size of the nanoparticles are determined both by the nanotube core-forming peptide structure and the length and charge of the polymeric hydrophilic segment. In recent work, we demonstrated that a linear octapeptide formed by the regular (L-Ala-D-Val) repeat and end-linked to poly(ethylene glycol) (PEG) with 2 kDa molecular weight, was able to form well-defined core/shell nanorods the self-assembly of which was guided by the structure of the internal peptide channel [19]. The most interesting feature of this system is that they can be obtained starting from a linear oligopeptide that can be synthesised more easily than the cyclic analogue. De Santis and coworkers theoretically [6] and experimentally [7] demonstrated that β-like structures are stabilised above three ld-peptide units; consequently, hexapeptides with regular enantiomeric sequences can form β-helices similar to that formed by octapeptides. In contrast,

* Corresponding author.
E-mail address: giancarlo.masci@uniroma1.it (G. Masci).
[1] Present address: IRBM Science Park S.p.A., Via Pontina Km 30.600 - 00040 Pomezia (RM), Italy.

http://dx.doi.org/10.1016/j.nbt.2016.07.005
1871-6784/



Scheme 1. Reaction scheme for synthesis of the conjugates.

shorter peptides such as tetrapeptides adopt a Bragg-type hydrogen-bonded $\pi_{L,D}$ conformation similar to a β-bend structure [7]. Moreover, it is well known that the ratio of the solvophilic and solvophobic segments in block copolymers significantly affects the morphology and stability of the nanoparticles formed by self-assembly [20].

For these reasons, in this paper we report the synthesis and structural characterisation of two conjugates formed by end-linking linear tetra- and hexapeptides Cbz-(L-Ala-D-Val)$_n$-OH with n = 2 and 3 and Cbz = carbobenzyloxy, obtained by solid phase peptide synthesis (SPPS), to an amine-end functionalised poly (ethylene glycol) chain. The self-assembling capability of the conjugates was investigated by circular dichroism, fluorescence spectroscopy, ^{1}H NMR, scanning electron microscopy and dynamic light scattering. The drug loading end release capacity of the model drug curcumin was also studied.

Experimental section

Materials and methods

Materials

Dichloromethane (DCM), acetonitrile (MeCN, Chromasolv Plus, for HPLC), diethyl ether, hexane, *N*,*N*-dimethylformamide (DMF), trifluoroacetic acid (TFA), 1-methyl-2-pyrrolidone (NMP), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU), *N*,*N*-diisopropylethylamine (DIPEA), thionyl cloride ($SOCl_2$), triethylamine (TEA), sodium azide, *n*-buthanol, 4-(dimethylamino)pyridine (DMAP), all peptide synthesis grade were purchased from Aldrich and used as provided. Spectrograde pyrene and palladium on activated carbon (10 wt% Pd/C) were from Fluka. Piperidine (Fluka) was distilled before use. DCM and DMF were dried with 4 Å molecular sieves. Fmoc-D-Val-Wang resin (loading level 0.7 mmol g^{-1}, 1% DVB, 200–400 mesh, Fmoc = 9-fluorenylmethoxycarbonyl) was purchased from Novabiochem. L-Ala and D-Val Fmoc-amino acids and Cbz-L-Ala-OH were from Aldrich. Analytical standard curcumin, phosphate buffered saline powder (PBS, pH 7.4), butylated hydroxyanisole (BHT) and TWEEN® 20 for molecular biology were from Aldrich. Deuterated solvents for NMR spectroscopy were used as obtained from Aldrich. Spectrograde ethanol and trifluoroethanol (TFE) were purchased from Sigma and used as provided. Methoxy poly(ethylene glycol) (mPEG) (M_n = 2000, M_w/M_n = 1.03, Aldrich) was dried in a vacuum oven at 80 °C for 2 h before use. Thin Layer Chromatography (TLC) was performed on silica gel Merck 60 F254 plates.

Synthesis of cbz-(L-Ala-D-Val)$_n$-OH (n = 2, 3). general procedure

The linear peptides, Cbz-(L-Ala-D-Val)$_2$-OH (PEP4) and Cbz-(L-Ala-D-Val)$_3$-OH (PEP6), were prepared by conventional SPPS on 400 mg of Fmoc-D-Val-Wang resin (280 μmol scale) as described in the literature [21]. The synthesis of both peptides is reported in Scheme 1. In both cases, coupling was obtained for reaction of 2 equivalents of Fmoc-L-Ala-OH, Fmoc-D-Val-OH and Cbz-L-Ala-OH-OH, TBTU, and DIPEA in NMP (5 mL). The reaction mixtures were incubated for 3 h at room temperature (RT) with mechanical shaking. The ninhydrin-Kaiser test was used to assess completion of the reaction. The Fmoc protecting group was removed in 20% piperidine solution in DMF (2 × 5 mL, 15 min). 95% TFA aqueous solution (2 mL, 3 h) was used to cleave the final peptide from resin. The peptide was precipitated with dry diethyl ether/hexane 4/6 at 0 °C. After repeated washing with ether/hexane 4/6, the solid was solubilised in 1/1 dioxane/water and finally freeze dried. A semi-preparative liquid chromatography system (Waters 600E) with a Waters μBondapak C-18 column (1.9 × 30 cm, 5 μm, 300 Å) was used to purify the crude peptides (flow rate 8 ml min^{-1}, 10–60% linear gradient of MeCN in 0.1% aqueous TFA in 30 min). The final peptide purity was >98% as determined by analytical HPLC. The peptides were characterised by monodimensional ^{1}H NMR spectroscopy and ESI–MS spectrometry. ^{1}H NMR spectra of PEP4 in $CDCl_3$ and PEP6 in DMSO-d_6 and ESI–MS spectra of both peptides in methanol were reported in Supplementary material (Figures S.1-S.4).

PEP4 Yield: 0.132 g (96%). ^{1}H NMR ($CDCl_3$, 300 MHz), δ (ppm): 8.10-7.44 (m, 4H, —N*H*—CO—), 7.31 (s, 5H, C_6H_5-), 5.22-4.92 (m, 2H —C*H*$_2$—C_6H_5), 4.91-4.16 (m, 4H, α-C*H*), 2.31-1.88 (m, 2H, β-C*H*-Val), 1.50-1.25 (m, 6H, C*H*$_3$-Ala), 0.94 (s, 12H, (C*H*$_3$)$_2$—CH-Val).

ESI–MS for $C_{24}H_{36}N_4O_7$ (calculated 492.21), *m*/*z* (%): 493.34 (24) [M + H]$^+$, 515.24 (100) [M + Na]$^+$, 531.24 (62) [M + K]$^+$.

PEP6 Yield: 0.174 g (94%). ^{1}H NMR (DMSO-d_6, 300 MHz), δ (ppm): 8.68-7.41 (m, 6H, —N*H*-CO), 7.34 (s, 5H, C_6H_5-), 5.01 (s, 2H